Functional Recovery in Aged and Young Rats After Embolic Stroke

Treatment With a Phosphodiesterase Type 5 Inhibitor

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Background and Purpose—Advanced age is associated with a decrease in brain plasticity compared with the young adult. Sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor promotes brain plasticity and improves functional outcome after stroke in the young animal. Here, we test the hypothesis that sildenafil provides restorative therapeutic benefit to the aged animal.

Methods—Male Wistar rats (aged, 18-month old; young, 3-month old) were subjected to embolic stroke. Saline or sildenafil was administered daily at a dose of 2 mg/kg orally or 10 mg/kg subcutaneously for 7 consecutive days starting 24 hour after stroke onset.

Results—Aged rats exhibited significant impairment of functional recovery and reductions of vascular density, and endothelial cell proliferation compared with young rats. Aged rats treated with sildenafil at a dose of 10 mg/kg but not 2 mg/kg, showed significant improvements of functional recovery and concomitant increases in cortical cyclic guanosine 3′,5′-cyclic monophosphate (cGMP) level, vascular density, endothelial cell proliferation, and synaptogenesis compared with aged rats treated with saline. In young rats, treatment with sildenafil at a dose of 2 or 10 mg/kg significantly enhanced functional recovery and amplified brain plasticity compared with young rats treated with saline.

Conclusion—Age is associated with reduction of angiogenesis, and poor neurological functional recovery after stroke. However, treatment of aged stroke rats with sildenafil improves functional recovery that is likely fostered by enhancement of angiogenesis and synaptogenesis. (*Stroke*. 2005;36:847-852.)

Key Words: angiogenesis ■ embolism ■ recovery of function

Phosphodiesterase type 5 (PDE 5) enzyme is highly specific for hydrolysis of cGMP and regulates cGMP signaling.[1,2] Administration of an NO donor elevates cerebral cGMP level, and improves neurological functional recovery in young rats after stroke.[3] In normal rats, administration of sildenafil, an inhibitor of PDE 5 elevates cortical cGMP level.[4] Treatment of stroke with sildenafil improves neurological functional recovery in young rats.[4]

Aged rats exhibit a decrease in the basal brain levels of cGMP[5] which may have important functional implications such as, for learning and memory.[5] Thus, the efficacy of sildenafil treatment of stroke in the aged animal may significantly decline with aging, which may have important clinical implications for stroke treatment because stroke is a major cause of death and disability in the elderly.[6] Accordingly, in the present study, we test the hypothesis that treatment of stroke with sildenafil improves neurological functional recovery in aged rats after stroke.

Materials and Methods

Male Wistar rats at ages of 8 to 12 weeks (Charles River Breeding Co, Wilmington, Mass) and 18 months (Harlan Winkelmann GMBH, Germany) were classified as young and aged, respectively. Rats were individually housed in standard Plexiglas laboratory cages (550×350×360 mm) within a large well-ventilated room with a constant temperature of 23°C with a 12-hour light/dark cycle, and free access to food and water. Sildenafil (Viagra, Pfizer Inc) is a weak basic compound, which has a half-life of 0.4 hour in male rats.[7]

Animal Model

The MCA was occluded by placement of an embolus at the origin of the MCA, as previously described.[8] All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Experimental Protocol

To examine the effect of sildenafil on aged rats, sildenafil was administered at a dose of 2 mg/kg (n=10) orally (PO) or 10 mg/kg (n=8) subcutaneously (SQ) to rats 24 hours after MCA occlusion and daily for an additional 6 days. Aged stroke rats (n=15) were treated with the same volume of saline as the control group. To

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TABLE 1. Mortality Rate and Infarct Volume

	Mortality Rate, %	Infarct Volume (% of Hemisphere)
Young		
Saline	9	38.8±12.2
Sildenafil, 2 mg/kg	10	38.1±10.5
Sildenafil, 10 mg/kg	8	36±9.2
Aged		
Saline	47*	32.8±14.8
Sildenafil, 2 mg/kg	30	33.1±9.3
Sildenafil, 10 mg/kg	13	30±0.2

Values are mean±SE.
*P<0.05 vs respective young groups.

examine the effect of sildenafil on young rats, sildenafil was administered at a dose of 2 mg/kg (n=10, PO) or at a dose of 10 mg/kg (n=13, SQ) to rats 24 hour after MCA occlusion and daily for an additional 6 days. Young stroke rats (n=12) treated with the same volume of saline SQ were used as the control group. The oral dosing protocol of 2 mg/kg was previously used the young rats.[3]

Plasma Concentration of Sildenafil

Blood was sampled from young rats at 1 hour after 2-mg/kg (n=3, PO) and 10-mg/kg (n=4, SQ) doses of sildenafil on days 1, 4 and 7 of treatment. Sildenafil plasma concentration analysis was carried out by Pfizer Global Research and Development.

Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU) was used for mitotic labeling. Animals received intraperitoneal injections of BrdU (100 mg/kg, Sigma) twice a day starting at 24 hour after stroke and subsequently for 7 consecutive days.

Functional Outcome

All functional outcome tests were performed by observers blinded to the treatments pre-ischemia, and at 1, 7, 14, 21, and 30 days after onset of MCA occlusion.

Neurological Severity Score

Neurological Severity Score (NSS) is a composite of motor, sensory, reflex, and balance tests.[9] Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Adhesive Removal Test

An adhesive removal test was used to measure somatosensory deficits.[10] The mean time required to remove both stimuli from limbs was recorded.

Foot-Fault Test

Rats were tested for placement dysfunctions of forelimbs with the modified foot-fault test.[11] The total number of steps (movement of each forelimb) that the rat used to cross the grid and the total numbers of foot faults for each forelimb were recorded.

Corner Test

Rats were tested for vibrissae sensory, postural, and motor asymmetries with the corner test.[12] The number of ipsilateral (right) turns was recorded from 10 trials for each test.

Histopathologic Studies

At 30 days after MCA occlusion, each rat was transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde. Infarct volume was measured on 7 hematoxylin and eosin (H&E) stained coronal sections using a Global Laboratory Image analysis program (Data Translation), as previously described.[9]

Immunohistochemistry and Quantification

For morphological analysis of vessels, a monoclonal antibody (mAb) against vWF (DAKO, Glostrup, Denmark) was used at a titer of 1:400. For measurement of cerebral vascular density, 2 vWF immunostained coronal sections (6 um) at bregma -0.2, and -2.8 mm were digitized using a 20x objective via the MicroComputer Imaging Device system. The numbers of vessels were counted throughout the ischemic boundary area. The total number of vessels was divided by the total boundary area to determine vascular density.

For BrdU immunostaining, a mAb against BrdU (Boehringer Mannheim) was used at a titer of 1:1000. To quantify BrdU immunoreactive endothelial cells, numbers of endothelial cells and numbers of BrdU immunoreactive endothelial cells in 10 enlarged vessels adjacent to the ischemic lesion and 10 vessels of the contralateral homologous area were counted from each rat. Data are presented as a percentage of BrdU immunoreactive endothelial cells to total endothelial cells in 10 enlarged vessels from each rat.

To detect presynaptic plasticity and synaptogenesis, a mAb antisynaptophysin (Boehringer Mannheim) was used at a titer of 1:500.[13] For quantification of synaptophysin immunoreactivity, 2 immunostained coronal sections (bregma -0.2, and -2.8 mm) and 8 fields of view from the ischemic boundary area and the contralateral homologous area in each section were digitized under a 20x objective. The synaptophysin immunoreactive area was measured. Data are presented as a percentage compared with the contralateral homologous region on the same section.

cGMP Measurement in Brain Tissue

Male Wistar rats at ages of 8 to 12 weeks (n=8) and 15 months (n=4; Charles River Breeding Co, Wilmington, Mass) were used to examine the effects of age and sildenafil on brain cGMP levels at 7

TABLE 2. Neurological Functional Tests

	NSS (Scores)					Adhesive Removal Test (s)				
	1 d	7 d	14 d	21 d	30 d	1 d	7 d	14 d	21 d	30 d
Young										
Saline	10±0.3	8.3±0.3	7.1±0.7	6.3±0.4	5.3±0.4	120±0.1	102±9.7	64.8±8.4	43.5±6.4	30.1±4.1
2 mg/kg	10.2±0.4	7.7±0.4	6.2±0.5	4.9±0.3*	3.4±0.5*	120±0.1	78±9.8	36±6.3*	21±3.0*	13±2.1*
10 mg/kg	11.1±0.4	7.0±0.3*	5.0±0.3*	3.3±0.4*	2.4±0.3*	120±0.1	80±7.0	58±6.0*	24±2.5*	16±1.8*
Aged										
Saline	11.5±0.5†	8.8±0.5	7.9±0.7	6.5±0.6	5.8±0.7	120±0.1	100±7.4	100±8.4†	81±9.3†	59±8.1†
2 mg/kg	11.3±0.5	8.1±0.4	6.7±0.3	5.4±0.2	4.0±0.2*	120±0.1	113±4.5	100±9.9	73±10	40±4.1
10 mg/kg	11.4±0.6	7.4±0.4	5.7±0.4*	4.5±0.5*	3.6±0.4*	120±0.1	102±8.0	88±8.8	52±0.4*	28±3.5*

Values are mean±SE.
*P<0.05 vs respective saline treated groups.
†P<0.05 vs saline treated young rats.

days after stroke, respectively. The ipsilateral hemisphere cortical tissue was rapidly removed and dissected. Levels of cGMP were measured with the use of a commercially available low-pH immunoassay kit (R&D Systems Inc) according to the manufacturer's instruction.

Statistics

Two-way analysis of variance (ANOVA) was used to test overall treatment and age effects of ordinal data between groups. Logistic regression analysis was used to test the mortality rate among the groups. All values are presented as mean ± SE. Statistical significance was set at $P<0.05$.

Results

Mortality

A significant increase of mortality rate ($P<0.05$) was detected in aged rats compared with young rats after saline treatment (Table 1). Rats died between 2 and 24 days after stroke onset. No animals died during or immediately after induction of cerebral ischemia, or immediately after drug administration. Rats that died were excluded from further evaluation.

Lesion Volume

There was no significant difference of infarct volume among the groups (Table 1).

Neurological Functional Outcome

After saline treatment, aged rats exhibited a significantly greater impairment of neurological function measured by NSS at 1 day, and by the adhesive removal test at 14, 21, and 30 days after stroke compared with young rats. However, no significant difference of the foot-fault test score and corner test score was detected among saline treated aged and young rats after stroke (Table 2). In aged rats, treatment with sildenafil at a dose of 10 mg/kg SQ, but not 2 mg/kg PO, significantly ($P<0.05$) improved performance on NSS and adhesive removal test compared with the saline treated rats (Table 2). However, no significant difference of the foot-fault test score and corner test score was detected among aged groups (Table 2). In young rats, treatment with sildenafil at a dose of 2 mg/kg PO and 10 mg/kg SQ significantly ($P<0.05$) improved performance on NSS, adhesive removal test, foot-fault test, and corner test compared with the saline treated rats (Table 2).

TABLE 3. Sildenafil Plasma Concentrations

Groups	1 d	4 d	7 d
Sildenafil, 2 mg/kg	0.7*	4.1†	2.1*
Sildenafil, 10 mg/kg	34.3±5.9	33.4±5.0	100.8±12.0

Values are free plasma concentrations in ng/ml, and are presented as mean±SE. Sildenafil concentration below limit of detection (0.5 ng/ml) in *samples, and in †samples.

Sildenafil Plasma Concentrations

Free plasma concentrations of sildenafil in young rats at 1 hour after dosing on days 1, 4 and 7 were between 0.7 and 4.1 ng/mL with 2 mg/kg PO treatment, and between 33.4 to 100.8 ng/mL with 10 mg/kg SQ treatment, respectively (Table 3).

Effects of Sildenafil on Vascular Density and Endothelial Proliferation

After stroke, the saline treated aged rats exhibited significantly lower vessel density and less endothelial proliferation within the ipsilateral hemisphere compared with saline treated young rats (Figure 1). Treatment with sildenafil at a dose of 10 mg/kg SQ significantly ($P<0.05$) increased the vessel density and endothelial proliferation within the ipsilateral hemisphere in both aged and young rats compared with their respective saline treated rats. (Figure 1).

Effects of Sildenafil on Synaptophysin

After stroke, no significant difference of the synaptophysin immunoreactivity was detected among saline treated aged and young rats. Treatment with sildenafil at a dose of 10 mg/kg SQ significantly ($P<0.05$) increased the percentage of synaptophysin immunoreactive area in aged and young rats compared with their respective controls (Figure 2).

Effects of Sildenafil on Cortical cGMP Level

Saline treated aged rats exhibited a significantly ($P<0.05$) lower level of cortical cGMP compared with saline treated young rats at 7 days after MCA occlusion. Treatment with sildenafil 10 mg/kg SQ significantly ($P<0.05$) increased the cortical cGMP level by 30% in aged rats, and 60% in young rats compared with saline treated aged and young rats, respectively (Figure 3).

Discussion

The present study demonstrates that aged rats exhibited impairment of functional recovery and angiogenesis after

TABLE 2. Continued

Foot-Fault Test (% of Errors)					Corner Test (No. of Right Turns)				
1 d	7 d	14 d	21 d	30 d	1 d	7 d	14 d	21 d	30 d
38.6±1.8	27.4±1.3	23.4±1.2	18.9±1.1	15.8±1.0	10± 0.0	9.0±0.3	7.6±0.4	6.8±0.2	6.3±0.2
35.8±1.1	27.1±1.3	18.7±1.5*	14±1.2*	10.2±1.0*	10±0.0	7.9±0.4	6.9±0.3	6±0.4*	5.3±0.4*
38.4±1.3	25.1±1.2	18.7±1.4*	13.1±1.4*	8.9±0.7*	9.0±0.2	8.1±0.3	6.7±0.2	5.0±0.3*	5.4±0.2*
38.6±1.6	31±1.7	28.3±1.5	21.5±1.5	19±1.7	9.9±0.1	9.4±0.4	8.1±0.5	6.0±0.4	6.0±0.3
39.4±1.0	30.3±1.5	25.1±1.7	19.7±1.2	16.0±1.0	9.0±0.4	8.7±0.4	7.4±0.2	6.4±0.2	6.1±0.1
37.7±1.3	26.3±1.9	22±1.6	18.6±1.0	14±1.4	9.8±0.1	8.8±0.4	7.3±0.4	6.1±0.3	5.9±0.3

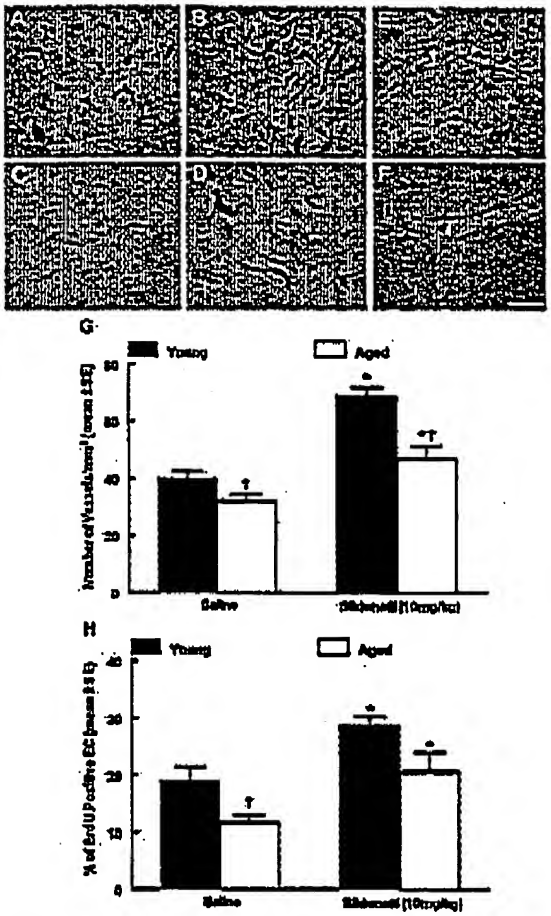

Figure 1. Vascular density and proliferated cerebral endothelial cells. Panels A through D show vWF immunoreactivity in the ischemic boundary area of representative young rats (A, B) and aged (C, D) treated with saline and sildenafil (10 mg/kg, SC) at 30 days after MCA occlusion, respectively. Panels E and F show BrdU immunoreactive endothelial cells (arrows) in an enlarged thin-wall vessel of representative young and aged rats treated with sildenafil (10 mg/kg, SC). Treatment with sildenafil (10 mg/kg, SC) significantly increased the vessel density within ischemic boundary area (G) and numbers of proliferated endothelial cells (H). *$P<0.05$ versus the saline treated respective groups. †$P<0.05$ versus respective young groups. Bar in A through F=50 μm.

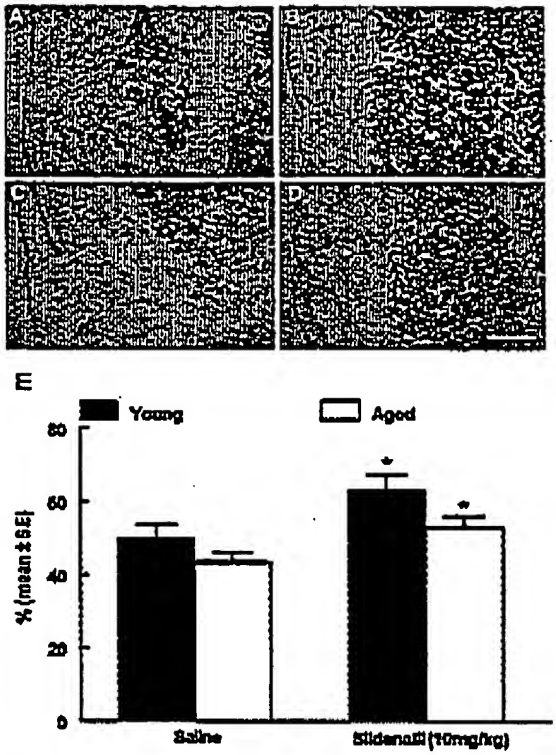

Figure 2. Synaptophysin immunoreactivity. Panels A through D show synaptophysin immunoreactivity in the ischemic boundary area of representative young rats (A, B) and aged (C, D) treated with saline and sildenafil (10 mg/kg, SC) at 30 days after MCA occlusion, respectively. Bar in A through D=200 μm. Treatment with sildenafil (10 mg/kg, SC) significantly increased the percentage of synaptophysin immunoreactive area within ischemic boundary compared with the controls (E). *$P<0.05$ versus the saline treated respective groups.

embolic stroke and that treatment with sildenafil improves functional recovery that was associated with enhancement of angiogenesis and synaptogenesis around the ischemic boundary regions. Thus, aged brain has the capacity to enhance plasticity in response to sildenafil treatment.

Although histological analysis showed that infarct volume in the aged rat is comparable with the data obtained from the young rats, a significantly higher mortality rate and more severe neurological functional impairments, ie, adhesive removal test and NSS, were detected in aged versus young rats. Our data are in good agreement with clinical findings and previous experimental studies.[6,14,15,16] Thus, our data demonstrate that aging is an important determinant of outcome after embolic stroke. The different patterns of age associated functional outcome measured by NSS and the adhesive removal test suggests that these outcome measurements target different aspects of functional outcome after ischemia. Adhesive removal test measures somatosensory dysfunction, whereas NSS measures an amalgamation of motor, sensory, reflex, and balance outcome.[9,10] Adhesive removal times in saline treated aged rats were significantly higher than in young rats during 14 to 30 days after stroke, whereas a significant difference on NSS was only detected at day 1 after stroke, suggesting that the adhesive removal test is a more sensitive indicator of aged related functional impairments after stroke. The lack of significant differences of functional outcome between aged and young rats measured by foot-fault test and corner test suggests that these two tests are not sufficiently sensitive to assess age related functional deficits after stroke.

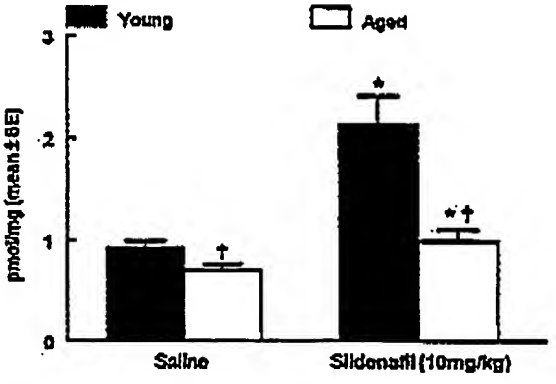

Figure 3. Ipsilateral cortex cGMP Levels in aged and young rats treated with saline and sildenafil (10 mg/kg, SC) at 7 days after MCA occlusion. *P<0.05 versus the saline treated respective groups. †P<0.05 versus respective young groups.

Aging is associated with reduced expression of vascular endothelial growth factor (VEGF) and impairment of angiogenesis, which may result in poor functional recovery after stroke.[17,18] The restoration of cerebral circulation after angiogenesis is important in the ischemic brain for functional recovery after a stroke.[19] In the central nervous system, NO/cGMP signaling promotes angiogenesis, neurogenesis, axonal outgrowth, and synaptic plasticity during development and in the adult animal.[20,21,22] However, advanced age is associated with impairment of NO/cGMP pathway, which may result in poor brain plasticity.[3] In the experimental model of stroke, administration of sildenafil and an NO donor, DETANONOate, increases brain levels of VEGF and angiogenesis in the ischemic brain, suggesting that cGMP contributes to NO-induced VEGF synthesis.[4] Specific cGMP-dependent protein kinase type I knockout mice exhibit strongly reduced cerebellar long-term depression of synaptic transmission, which suggest that cGMP is involved in cerebellar synaptic plasticity.[23] In the present study, cortical cGMP level and angiogenesis are significantly reduced in aged rats compared with young rats after stroke. Our data suggest that the impairment of functional recovery in aged rats after stroke are presumably because of the reduction of angiogenesis as a consequence of age-related reduction of cGMP production.

In the present study, treatment with sildenafil at a dose of 10 mg/kg SQ increased angiogenesis, synaptogenesis, and improved neurological functional recovery in both young and aged rats. However, treatment with sildenafil at a dose of 2 mg/kg PO significantly improved functional recovery in young rats, but failed to show improvement in aged rats. Plasma concentrations of sildenafil were above the levels associated with therapeutic efficacy in humans (5 to 15 ng/mL) for at least 1 hour after dosing with 10 mg/kg SQ in young rats, but below these levels after 2 mg/kg PO even though neurological function was also improved by this dose of sildenafil.[24] Although plasma concentrations were not measured in aged rats, sildenafil concentrations were likely to have been higher in the aged rats (unpublished data, Pfizer Global Research and Development) suggesting that pharmacokinetic differences were not responsible for the lack of effect at 2 mg/kg PO. Moreover, administration of sildenafil at a dose of 10 mg/kg SQ resulted in a 30% increase of cortical cGMP levels in aged rats at 7 days after stroke, whereas a 60% increase was detected in young rats. Collectively, our data suggest that although limited, the aged brain retains the capacity to increase cGMP level in response to sildenafil treatment, and to subsequently enhance angiogenesis and synaptic plasticity. The failure of functional improvement after low-dose sildenafil treatment (2 mg/kg, PO) in aged animal is likely attributed to the impaired endogenous brain plasticity in the aged versus young rats (see Figure 1, 2).

In summary, present study demonstrates that age is associated with impairment of angiogenesis after stroke. Treatment with sildenafil improves neurological functional, and enhances brain plasticity in young and aged rats after MCA occlusion.

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